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60/000,449 PROVISIONAL	06/22/95				

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TITLE EOTAXIN: AN EOSINOPHIL CHEMOATTRACTANT

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APPROVED

PATENT APPLICATION



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# PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53 (b)(2).

Docket Number		00383/025001		Type a plus sign (+) inside this box →
INVENTOR(s)/APPLICANT(s)				
LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)	
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TITLE OF THE INVENTION (250 characters max)				
EOTAXIN: AN EOSINOPHIL CHEMOATTRACTANT				
CORRESPONDENCE ADDRESS				
Fish & Richardson 225 Franklin Street Boston, MA 02110-2804				
STATE	MA	ZIP CODE	02110-2804	COUNTRY USA
ENCLOSED APPLICATION PARTS (check all that apply)				
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METHOD OF PAYMENT (check one)				
<input type="checkbox"/> A check or money order is enclosed to cover the Provisional filing fee			PROVISIONAL FILING FEE AMOUNT (\$)	\$75.00
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☐ No.

☐ Yes, the name of the U.S. Government agency and the Government contract number are: \_\_\_\_\_

Respectfully submitted,

SIGNATURE

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Date

6/22/95

REGISTRATION NO. (if appropriate)

39,109



Additional inventors are being named on separately numbered sheets attached hereto

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## PROVISIONAL APPLICATION

UNDER 37 CFR 1.53(b)(2)

TITLE: EOTAXIN: AN EOSINOPHIL CHEMOATTRACTANT

APPLICANTS: ANDREW D. LUSTER, PHILIP LEDER, MARC ROTHENBERG,  
AND EDUARDO GARCIA

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Mary Jane D. Palma



A/PROVISIONAL

PATENT  
ATTORNEY DOCKET NO: 00383/025001

EOTAXIN: AN EOSINOPHIL CHEMOATTRACTANT

Statement as to Federally Sponsored Research

5 This work was funded in part by NIH grant DK43351.

The Field of the Invention

The invention relates to regulation of the immune system.

Background of the Invention

10 The chemokines are a family of 8-12 kD proteins that regulate leukocyte trafficking by binding to specific seven transmembrane spanning G-protein-linked receptors. They can be divided into three families depending upon the sequence of conserved cysteine residues and this structural  
15 distinction corresponds to specific biologic properties in that the C-X-C, C-C, and C families are mainly chemoattractive for neutrophils, monocytes, and lymphocytes, respectively. Additionally, each chemokine family maps to a different chromosomal locus.

20 Eosinophils are circulating leukocytes that are thought to dwell predominantly in tissues where they survive for several weeks and mediate pro-inflammatory and cytotoxic damage in selected diseases (e.g. asthma, parasitic infections, and malignancy). Given their presumed role in  
25 the pathogenesis of inflammatory states, the regulation of tissue recruitment of eosinophils is of interest and various chemoattractants have been found to be active on eosinophils, including leukotriene B<sub>4</sub>, platelet activating factor (PAF), and several chemokines Resnick, M. B. &  
30 Weller, P. F. (1993) Amer. J. Resp. Cell. Mol. Biol. 8, 349-355. Chemokines active on eosinophils include certain C-C chemokines: monocyte chemoattractive protein (MCP)-2 and 3, RANTES, and macrophage inflammatory protein (MIP)-1 $\alpha$  Rot,

A., Krieger, M., Brunner, T., Bischoff, S. C., Schall, T. J. & Dahinden, C. A. (1992) J. Exp. Med 176, 1489-1495; Alam, R., Stafford, S., Forsythe, P., Harrison, R., Faubion, D., Lett-Brown, M. A. & Grant, J. A. (1993) J. Immun. 150, 3442-3448; Dahinden, C. A., Geiser, T., Brunner, T., Vontscharner, V., Caput, D., Ferrara, P., Minty, A. & Baggiolini, M. (1994) J. Exp. Med. 179, 751-756; Weber, M., Ugucioni, M., Ochensberger, B., Baggiolini, M., Clark-Lewis, I. & Dahinden, C. A. (1995) J. Immun. 154, 4166-4172. A C-X-C chemokine, interleukin-8 (IL-8), is also chemoattractive for cytokine-primed eosinophils (11). Notwithstanding their activity, none of these chemoattractive molecules are eosinophil specific and their relative importance in selected diseases and in experimental animal models of allergy remains unclear.

#### Summary of the Invention

In general, the invention features substantially pure DNA (for example, genomic DNA, cDNA, or synthetic DNA) encoding an eotaxin polypeptide as defined below. In related aspects, the invention also features a vector, a cell (e.g., a bacterial, yeast, nematode, or mammalian cell), and a transgenic animal which includes such a substantially pure DNA encoding an eotaxin polypeptide.

In preferred embodiments, the eotaxin gene is the human eotaxin gene provided in Fig. 14. In various preferred embodiments, the cell is a transformed animal cell.

In related aspects, the invention features a transgenic animal containing a transgene which encodes an Eotaxin polypeptide. The invention also features a cell that expresses the human eotaxin gene. Preferably, the cell is an animal cell which is a mucosal or endothelial cell.

In a second aspect, the invention features a substantially pure DNA which includes a promoter capable of expressing the eotaxin gene in a cell.

5 In preferred embodiments, the promoter is the promoter native to an eotaxin gene. Additionally, transcriptional and translational regulatory regions are preferably native to an eotaxin gene.

In another aspect, the invention features a method of detecting a eotaxin gene in a cell involving: (a)  
10 contacting the eotaxin gene or a portion thereof greater than 9 nucleic acids, preferably greater than 18 nucleic acids in length with a preparation of genomic DNA from the cell under hybridization conditions providing detection of DNA sequences having about 50% or greater sequence identity  
15 to the conserved DNA sequences of Fig. 14 or the sequences which are conserved among eotaxins relative to other proteins, as deduced from the polypeptide sequences provided in Fig. 3B. Preferably, the region of sequence identity used for hybridization is the region of 9 nucleic acids or  
20 more encoding the region of highest conservation between the sequences shown in Fig. 13 or among eotaxins in Fig 3B.

In another aspect, the invention features a method of producing an Eotaxin polypeptide which involves: (a)  
25 providing a cell transformed with DNA encoding an Eotaxin polypeptide positioned for expression in the cell (for example, present on a plasmid or inserted in the genome of the cell); (b) culturing the transformed cell under conditions for expressing the DNA; and (c) isolating the Eotaxin polypeptide.

30 In another aspect, the invention features substantially pure Eotaxin polypeptide. Preferably, the polypeptide includes a greater than 50 amino acid sequence substantially identical to a greater than 50 amino acid



sequence shown in the Fig. 12, more preferably the identity is to one of the conserved regions of homology shown in Fig. 12. The two or three amino acids immediately preceding the N-terminal most SVP sequence of amino acids may be any three amino acids, but preferably the terminal amino acids are HP, HPA, HPS, HPT or HPG, wherein the H is the N-terminal amino acid. Most preferably, the N-terminal amino acids are HP followed by SVP as shown in Fig. 14.

In another aspect, the invention features a method of regulating eosinophil chemotactic events wherein the method involves: (a) providing the eotaxin gene under the control of a promoter providing controllable expression of the eotaxin gene in a cell wherein the eotaxin gene is expressed in a construct capable of delivering an Eotaxin protein in an amount effective to alter the chemotactic events. The polypeptide may also be provided directly, for example, in cell culture and therapeutic uses. In preferred embodiments, eotaxin is delivered by expression of the eotaxin gene using a tissue-specific or cell type-specific promoter, or by a promoter that is activated by the introduction of an external signal or agent, such as a chemical signal or agent.

In a related aspect, the invention features a method of reducing inflammation and cytotoxic damage. For example, damage occurring during asthmatic reactions, parasitic infections, and malignancies. The method includes modulating eotaxin biological activities. Preferably, eotaxin activity is dependent using an agonist such as an anti-eotaxin antibody or eotaxin fragment.

In yet another related aspect, the invention features a method of improving prognosis in patients with tumors. The method includes providing eotaxin in the region of the tumor either by providing and eosinophil attracting

amount of the polypeptide or by providing an eosinophil-attracting amount of a transgene expressing the polypeptide.

In other aspects, the invention features a substantially pure oligonucleotide including one or a combination of the sequences shown in Figs. 3A and 14A-14F.

In a another aspect, the invention features a method of isolating a eotaxin gene or fragment thereof from a cell, involving: (a) providing a sample of cellular DNA; (b) providing a pair of oligonucleotides having sequence homology to a conserved region of an eotaxin gene (for example, the oligonucleotides of Fig. 14); (c) combining the pair of oligonucleotides with the cellular DNA sample under conditions suitable for polymerase chain reaction-mediated DNA amplification; and (d) isolating the amplified eotaxin gene or fragment thereof. Where a fragment is obtained by PCR standard library screening techniques may be used to obtain the complete coding sequence.

In preferred embodiments, the amplification is carried out using a reverse-transcription polymerase chain reaction, for example, the RACE method.

In another aspect, the invention features a method of identifying a eotaxin gene in a cell, involving: (a) providing a preparation of cellular DNA (for example, from the human genome); (b) providing a detectably-labelled DNA sequence (for example, prepared by the methods of the invention) having homology to a conserved region of an eotaxin gene; (c) contacting the preparation of cellular DNA with the detectably-labelled DNA sequence under hybridization conditions providing detection of genes having 50% or greater sequence identity; and (d) identifying an eotaxin gene by its association with the detectable label.

In another aspect, the invention features a method of isolating an eotaxin gene from a recombinant DNA library,

involving: (a) providing a recombinant DNA library; (b) contacting the recombinant DNA library with a detectably-labelled gene fragment produced according to the PCR method of the invention under hybridization conditions providing  
5 detection of genes having 50% or greater sequence identity; and (c) isolating a member of an eotaxin gene by its association with the detectable label.

In another aspect, the invention features a method of isolating an eotaxin gene from a recombinant DNA library,  
10 involving: (a) providing a recombinant DNA library; (b) contacting the recombinant DNA library with a detectably-labelled Eotaxin oligonucleotide of the invention under hybridization conditions providing detection of genes having 50% or greater sequence identity; and (c) isolating an  
15 eotaxin gene by its association with the detectable label.

In another aspect, the invention features a recombinant polypeptide capable of mediating eosinophil chemotactic events wherein the polypeptide includes a domain having a sequence which has at least 70% identity to at  
20 least one of the sequences of Fig. 3A or Fig. 12. Preferably, the identity is to the sequence in Fig. 12. More preferably, the region of identity is 80% or greater, most preferably the region of identity is 95% or greater.

In another aspect, the invention features an eotaxin  
25 gene isolated according to the method involving: (a) providing a sample of cellular DNA; (b) providing a pair of oligonucleotides having sequence homology to a conserved region of an eotaxin gene; (c) combining the pair of oligonucleotides with the cellular DNA sample under  
30 conditions suitable for polymerase chain reaction-mediated DNA amplification; and (d) isolating the amplified eotaxin gene or fragment thereof.

In another aspect, the invention features an eotaxin gene isolated according to the method involving: (a) providing a preparation of cellular DNA; (b) providing a detectably-labelled DNA sequence having homology to a conserved region of an eotaxin gene; (c) contacting the preparation of DNA with the detectably-labelled DNA sequence under hybridization conditions providing detection of genes having 50% or greater sequence identity; and (d) identifying an eotaxin gene by its association with the detectable label.

In another aspect, the invention features an eotaxin gene isolated according to the method involving: (a) providing a recombinant DNA library; (b) contacting the recombinant DNA library with a detectably-labelled eotaxin gene fragment produced according to the method of the invention under hybridization conditions providing detection of genes having 50% or greater sequence identity; and (c) isolating an eotaxin gene by its association with the detectable label.

In another aspect, the invention features a method of identifying an eotaxin gene involving: (a) providing a mammalian cell sample; (b) introducing by transformation (e.g. biolistic transformation) into the cell sample a candidate eotaxin gene; (c) expressing the candidate eotaxin gene within the cell sample or isolating eotaxin from the tissue sample or protein isolated therefrom; and (d) determining whether the cell sample elicits an alteration in eosinophil chemotaxis, whereby an increased eosinophil specific chemotactic increase identifies an eotaxin gene.

In another aspect, the invention features an eotaxin gene isolated according to the method involving: (a) providing a cell sample; (b) introducing by transformation into the cell sample a candidate eotaxin gene; (c)

expressing the candidate eotaxin gene within the tissue sample; and (d) determining whether the tissue sample elicits a eotaxin mediated response or decrease thereof, whereby a response identifies an eotaxin gene.

5 In another aspect, the invention features a purified antibody which binds specifically to an Eotaxin protein. Such an antibody may be used in any standard immunodetection method for the identification of an Eotaxin polypeptide. Such an antibody may also be used to inhibit eotaxin protein  
10 function and to predict prognosis in following tumor diagnosis.

In another aspect, the invention features a DNA sequence substantially identical to the DNA sequence shown in Fig. 14. In related aspects, the invention features DNA  
15 substantially identical to the DNA sequence shown in Fig. 3A.

In another aspect, the invention features a substantially pure polypeptide having a sequence substantially identical to an amino acid sequence shown in  
20 Fig. 12. As shown in Fig. 12, X may be any amino acid or be absent, but preferably X is selected from the group consisting of A, S, T, G, and the absence of an amino acid. More preferably, X is absent.

In another aspect, the invention features a kit for  
25 detecting compounds which modulate eotaxin mediated events. The kit includes Eotaxin encoding DNA positioned for expression in a cell capable of producing a detectable eotaxin response. By eotaxin response is meant those eotaxin mediated events described herein, particularly in  
30 the examples, below.

In a related aspect, the invention features a method for detecting a compound which alters eotaxin mediated events. The method includes: i) a cell having eotaxin

encoding DNA positioned for expression; ii) contacting said cell or extracts therefrom with the compound to be tested; iii) monitoring said cell or extracts therefrom for the ability to alter eotaxin mediated events, for example increased eosinophil chemotaxis.

By "eotaxin gene" is meant a gene encoding a polypeptide having the eosinophil chemoattractant characteristics or other eotaxin biological activities described herein below. An eotaxin gene is a gene having about 50% or greater sequence identity to at least one of the eotaxin sequences of Figs. 3A or 14, or a portion thereof. For example, the gene may encode human or murine eotaxin polypeptide. An eotaxin gene may also be defined as encoding a polypeptide with at least 50% of the activity of the murine eotaxin polypeptide described below (preferably, such a comparison done using assay components derived from the species from which the eotaxin polypeptide to be tested is derived.

By "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

By "substantially identical" is meant a polypeptide or nucleic acid exhibiting at least 50%, preferably 85%, more preferably 90%, and most preferably 95% homology to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison sequences will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity is typically measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications. Conservative substitutions typically include substitutions within the following groups: glycine alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

By a "substantially pure polypeptide" is meant an eotaxin polypeptide (or other polypeptide described herein) which has been separated from components which naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight eotaxin polypeptide. A substantially pure polypeptide may be obtained, for example, by extraction from a natural source (e.g., a mammalian cell); by expression of a recombinant nucleic acid encoding the polypeptide; or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., those described in column chromatography, polyacrylamide gel electrophoresis, or by HPLC analysis.

A protein is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural state. Thus, a protein which is chemically synthesized or produced in a

cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those derived from eukaryotic organisms but synthesized in *E. coli* or other prokaryotes.

By "substantially pure DNA" is meant DNA that is free of the genes which, in the naturally-occurring genome of the organism from which the DNA of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) a polypeptide described herein (for example, an eotaxin polypeptide).

By "positioned for expression" is meant that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of, e.g., an eotaxin polypeptide, a recombinant protein or a RNA molecule).

By "reporter fusion" is meant a hybrid polypeptide which includes functional portion of a protein whose presence may be assayed; such portions include, without limitation, alkaline phosphatase,  $\beta$ -glucuronidase (GUS), luciferase, chloramphenicol transacetylase (CAT), and  $\beta$ -galactosidase.



By "promoter" is meant minimal sequence sufficient to direct transcription. Also included in the invention are those promoter elements which are sufficient to render promoter-dependent gene expression controllable for cell-type specific, tissue-specific or inducible by external signals or agents; such elements may be located in the 5' or 3' regions of the native gene.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

By "transgene" is meant any piece of DNA which is inserted by artifice into a cell, and becomes part of the genome of the organism which develops from that cell. Such a transgene may include a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism.

By "transgenic" is meant any cell which includes a DNA sequence which is inserted by artifice into a cell and becomes part of the genome of the organism which develops from that cell. As used herein, the transgenic organisms are generally transgenic plants and the DNA (transgene) is inserted by artifice into the nuclear or plastidic genome.

By "conserved region" is meant any stretch of six or more contiguous amino acids exhibiting at least 30%, preferably 50%, and most preferably 70% amino acid sequence identity between two or more of the eotaxin family members.

By "detectably-labelled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule. Methods for detectably-labelling a

molecule are well known in the art and include, without limitation, radioactive labelling (e.g., with an isotope such as  $^{32}\text{P}$  or  $^{35}\text{S}$ ) and nonradioactive labelling (e.g., chemiluminescent labelling, e.g., fluorescein labelling).

5 By "transformation" is meant any method for introducing foreign molecules into a cell. For example, molecules may be introduced using velocity driven microprojectiles such as tungsten or gold particles. Such velocity-driven methods originate from pressure bursts which  
10 include, but are not limited to, helium-driven, air-driven, and gunpowder-driven techniques. Biolistic transformation may be applied to the transformation or transfection of a wide variety of cell types and intact tissues including, without limitation, intracellular organelles (e.g.,  
15 chloroplasts and mitochondria), bacteria, yeast, fungi, algae, and animal tissue.

By "purified antibody" is meant antibody which is at least 60%, by weight, free from proteins and naturally-occurring organic molecules with which it is naturally  
20 associated. Preferably, the preparation is at least 75%, more preferably 90%, and most preferably at least 99%, by weight, antibody, e.g., an eotaxin-specific antibody. A purified eotaxin antibody may be obtained, for example, by affinity chromatography using recombinantly-produced eotaxin  
25 protein or conserved motif peptides and standard techniques.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Detailed Description

30 The drawings will first be described.

FIG 1. Structure of murine eotaxin gene. A linear map of a 129SV genomic clone containing the eotaxin gene is

indicated. Exon 1 is within a 5 kb region between XhoI and EcoRI. The precise position has not been determined and this is indicated with the pair of vertical lines. Exon 2 & most of exon 3 are contained within a 1.4 kb EcoRV fragment. A repeat containing 18 CA dinucleotides is indicated between exons 2 and 3. Below is the corresponding mRNA with the positions of the start (ATG) codon, stop codon (TAA), and site for signal peptidase cleavage (arrow).

FIG 2. CA dinucleotide polymorphism and chromosomal mapping of murine eotaxin gene. (A) PCR primers flanking the (CA)<sub>n</sub> repeat were used to amplify DNA from the eotaxin 129SV clone (Figure 1), or genomic DNA from C57BL/6J, DBA/2J, or Mus. spretus. As a negative control, no DNA was added in the far right lane. The 100 bp ladder (left lane) was used as molecular weight markers. (B) Mouse chromosome 11 map. The right panel is the location of the eotaxin gene using the backcross panel (BSS) containing approximately 1500 loci (15). The left panel is the Chromosome Committee Consensus map (31). Other C-C chemokines are shown and referred to as small inducible cytokines, Scya1-6. Eotaxin has been assigned the name Scya11.

FIG 3. (A) Nucleotide sequence and predicted amino acid translation of full length murine eotaxin cDNA. The arrow indicates the predicted site for signal peptide cleavage. The underlined protein sequence corresponds to predicted mature eotaxin protein. The asterisks indicate the intron/exon borders. The hatched bars overlies the ATTTA sequences that has been reported to decrease mRNA stability. The open box indicates the poly adenylation signal. In (B), alignment of murine eotaxin protein with guinea pig eotaxin and other MCPs. The boxed amino acids are identical between different proteins. The position of the leader sequence cleavage site is indicated with the arrow. The conservation

of amino acid gaps in murine and guinea pig eotaxin are indicated with O\*O. The positions of lysine conservations are indicated with O+O.

5       FIG 4. Chemotactic response of murine eosinophils to murine eotaxin. The chemotactic response to eotaxin was determined using increasing concentrations of supernatant from eotaxin transfected J558L cells (A) or COS cells (B). As controls, eosinophils were exposed to buffer alone, PAF (10<sup>-7</sup> M), or 1000 ng/ml recombinant murine MIPl $\alpha$  (A). In  
10 each case, the supernatant from untransfected cells is shown as control. The results shown are representative of 3 experiments and presented as mean  $\pm$  range for replicate samples.

15       FIG 5. Northern analysis of total RNA from mouse organs. 10 mcg of total RNA from FVB/N (first nine lanes) and C57BL/6 mouse strains. Tissue source is indicated. High stringency hybridization with a full length eotaxin cDNA probe was performed and X-ray film was exposed for 1 day.

20       FIG 6. (A) Eotaxin mRNA following transplantation of IL-4 transfected tumors. IL4 transfected or untransfected control J558L tumor cells were s.c. injected in syngeneic Balb/c mice and RNA was extracted from the local skin tissue at various times. Eotaxin mRNA signals  
25 were normalized to the 28S mRNA signal. Each time point represents 3-7 mice and the values are expressed as mean  $\pm$  SEM. (B) Representative autoradiographic data from untreated mice (lanes a-e), or mice that were injected with untransfected tumor cells (CNTL) (lanes f-i) or IL-4  
30 transfected tumor cells (lanes j-m) 24 hrs prior to RNA extraction from skin samples. Eotaxin and 28S mRNA bands are shown from separate mice in each lane. An eotaxin cDNA probe that was limited to the coding region was used as a

probe and autoradiographs were exposed for 3 days. (C)  
Eotaxin mRNA levels in murine endothelial cells. 10 mcg of  
total RNA prepared from untreated endothelial cells (left  
lane) or following treatment with 200 U/ml recombinant  
5 murine IFN- $\gamma$  for 6 and 18 hrs, respectively. High  
stringency signals for murine eotaxin and MCP-1 after 3 days  
of film exposure are shown.

FIG 7. Nucleotide sequence and predicted amino acid  
translation of full length guinea pig eotaxin cDNA. The  
10 underlined protein sequence corresponds to the sequence of  
mature active eotaxin isolated from the bronchoalveolar  
fluid except for the boxed amino acids which were previously  
ambiguous (17). The arrow indicates the predicted site for  
signal peptide cleavage. The hatched bars overlies the ATTTA  
15 sequences that has been reported to decrease mRNA stability.  
These sequence data are available from EMBL/GenBank/DDBJ  
under accession number \_\_\_\_\_.

FIG 8. Guinea pig and mouse genomic analysis.  
Guinea pig genomic DNA was digested with EcoRI (Lane a) and  
20 PvuII (Lane b) and mouse genomic DNA was digested with EcoRV  
(Lane c). After electrophoresis and transfer to nylon  
membranes, a guinea pig eotaxin probe limited to the coding  
region of the cDNA was hybridized and washed under low  
stringency conditions. X-ray film was exposed for 2 weeks.  
25 Molecular weight markers (in kb) are shown to the left for  
lanes a & b and to the right for lane c.

FIG 9. Northern analysis of total RNA from various  
guinea pig organs. 10 mcg of total RNA was resolved in 1.5%  
agarose gels and transferred to nylon membranes. Tissue  
30 source is indicated. Splenocytes were treated with 2.5  
mcg/ml Con A for 48 hours. RNA from intestine and lung were  
isolated from two different animals. Hybridization and  
washing were performed under conditions of high stringency

using a guinea pig eotaxin probe that was limited to the coding region of the cDNA. X-ray film was exposed for 1 week.

FIG 10. Northern analysis of poly A RNA (2 mcg/lane) isolated from the lungs of guinea pigs challenged 3 hrs earlier with saline (lanes a-f) or OVA (lanes g-l). Each lane is RNA from a separate animal. Hybridization was performed with a guinea pig eotaxin probe (upper panel) or a guinea pig b-actin probe (lower panel). X-ray films were exposed for 24 hrs.

FIG 11. Eotaxin mRNA levels following OVA challenge. OVA sensitized or saline exposed guinea pigs were challenged with aerosolized OVA or saline, respectively, and eotaxin mRNA levels in the lungs were examined. The intensity of the band hybridization was determined by a Phosphor-Imager. The eotaxin mRNA expression is normalized to guinea pig b-actin mRNA expression in each sample. Data is represented in arbitrary units and the results are expressed as mean  $\pm$  SEM (n=5 or 6 animals for each group).

FIG 12 is the human eotaxin polypeptide sequence.

FIG 13 is a comparison of the human, guinea pig, and murine polypeptide sequences.

FIG 14A-F provides the Human eotaxin nucleic acid sequence. 14A is the sequence of Exon 2. 14B-D provide the sequence of intron 1 and exon 2. 14E and 14F provide the sequence of intron 2 and exon 3.

## I. INTRODUCTION

Unlike previously described chemokines active on eosinophils, eotaxin a recently described C-C chemokine, has been implicated as an eosinophil specific chemoattractant in a guinea pig model of allergic airway inflammation. Eotaxin appears to be unique among the chemokines since it causes

the selective infiltration of only eosinophils when injected into the skin and when directly administered to the lungs of naive guinea pigs. It has been unclear if guinea pig eotaxin represents a homologue of a previously known chemokine, or if eotaxin is a distinct chemokine. Furthermore, the biological significance of eotaxin in animal models of non-allergic disease has not been previously examined.

Using the guinea pig eotaxin cDNA as a molecular probe, we now provide murine and human eotaxin and examine the murine eotaxin biological properties. The following examples are to illustrate not limit the invention.

The structural similarities between mouse and guinea pig eotaxin indicate that both are more closely related to one another than to other members of the C-C family of chemokines. For example, each contains several unique features including a gap of two amino acids near the amino-end of the protein and the conservation of basic amino acids near the carboxy-end that distinguish it from other C-C chemokines. In sum, this comparison indicates that eotaxin is a distinct cytokine and not a homologue of one of the previously known members of this family. In addition, we have determined that eotaxin resides on mouse chromosome 11 in a region encoding other members of the C-C chemokine family. It is interesting that IL-4 and IL-5 also map to murine chromosome 11, however, these interleukins are not syntenic in man with the C-C chemokine locus (Lossie, A. C., Macphée, M., Buchberg, A. M. & Camper, S. A. (1994) Mamm. Gen. 5, S164-180).

We demonstrate here that murine eotaxin has biologic properties that are comparable to those of the guinea pig homologue. In order to demonstrate direct eosinophil chemotactic activity, we isolated eosinophils from IL-5

transgenic mice (Dent, L. A., Strath, M., Mellor, A. L. & Sanderson, C. J. (1990) J. Exp. Med. 172, 1425-1431) and measured eosinophil chemotaxis in-vitro. It is important to note that, despite the fact that there are large numbers of eosinophils in the hematopoietic organs of these IL-5 transgenic mice, there are few eosinophils in other tissues and these transgenic mice are quite healthy Dent, L. A., Strath, M., Mellor, A. L. & Sanderson, C. J. (1990) J. Exp. Med. 172, 1425-1431. This benign phenotype suggests that other signals (in addition to IL-5) are necessary for eosinophil tissue recruitment and activation. Using eosinophils from this system, we showed that recombinant mouse eotaxin is a powerful chemoattractant for eosinophils as are murine MIP-1a and PAF which were used as positive controls. No chemoattractive activity was seen for eotaxin on macrophages or neutrophils. However, these cells may have been desensitized since they had already migrated into an inflamed peritoneal cavity in-vivo. Neither MIP-1a or PAF are active solely on eosinophils making it difficult to use them to develop clinically useful antagonists against eosinophils (Resnick, M. B. & Weller, P. F. (1993) Amer. J. Resp. Cell. Mol. Biol. 8, 349-355; Rot, A., Krieger, M., Brunner, T., Bischoff, S. C., Schall, T. J. & Dahinden, C. A. (1992) J. Exp. Med 176, 1489-1495). However, the biological properties attributed to eotaxin in the guinea pig and now the mouse make it likely that there will be a similar activity and thus pharmacological utility, in humans. It is likely that the eosinophils isolated from the IL-5 transgenic mice have been primed in-vivo by IL-5 exposure and this potentiates their responsiveness to eotaxin. Consistent with this, IL-5 activates human eosinophils and primes them to respond to RANTES in-vitro (Rothenberg, M. E., Petersen, J., Stevens, R. L.,



Silberstein, D. S., McKenzie, D. T., Austen, K. F. & Owen, W. J. (1989) *J. Immun.* 143, 2311-2316; Ebisawa, M., Yamada, T., Bickel, C., Klunk, D. & Schleimer, R. P. (1994) *J. Immun.* 153, 2153-2160).

5       As would be expected, eotaxin mRNA is constitutively expressed in mucosal tissues wherein eosinophils normally reside (skin, lung and intestinal tract). Nonetheless, expression is also seen in thymus, lymph node, and muscle where resident eosinophils are rare. This pattern of mRNA  
10       tissue distribution is similar to that previously seen in guinea pig, although mice have higher expression in the thymus and skin while guinea pigs have higher expression in the lung (Rothenberg, M. E., Luster, A. D., Lilly, C. M., Drazen, J. M. & Leder, P. (1995) *J. Exp. Med.* 181,  
15       1211-1216). This is consistent with the fact that guinea pigs have high basal numbers of eosinophils in the lungs, while pulmonary eosinophils are undetectable in healthy mice. Such observations are further consistent with a role for eotaxin in promoting eosinophil pulmonary homing  
20       (Hutson, P. A., Church, M. K., Clay, T. P., Miller, P. & Holgate, S. T. (1988) *Am. Rev. Resp. Dis.* 137, 548-557).

      The expression of eotaxin in the skin has implications for human cutaneous disease since there are many disorders characterized by excessive infiltration and  
25       activation of skin eosinophils (e.g. atopic dermatitis, urticaria, bullous pemphigoid, etc.) (Leiferman, K. M. (1991) *J. Am. Acad. Derm.* 24, 1101-1112). The unexpected, but appreciable, expression of eotaxin mRNA in lymphoid tissue and muscle suggests that eotaxin may effect other  
30       cell types because eosinophils do not normally reside in these tissues. The expression in the thymus and lymph node suggests that eotaxin may direct lymphocyte homing. The expression we observe in the thymus may be related to the

constitutive expression of other chemokines in thymic epithelium (Gattass, C. R., King, L. B., Luster, A. D. & Ashwell, J. D. (1994) J. Exp. Med. 179, 1373-1378). Our experiments suggest that eotaxin mRNA is enriched in RNA prepared from thymic epithelium.

Without wishing to bind ourselves, we note that our studies also offer a potential explanation for the observation that eosinophils infiltrate a variety of human tumors, an observation that is generally associated with an improved prognosis for such patients (Iwasaki, K., Torisu, M. & Fujimura, T. (1986) Cancer 58, 1321-1327; Pretlow, T. P., Keith, E. F., Cryar, A. K., Bartolucci, A. A., Pitts, A. M., Pretlow, T. G. D., Kimball, P. M. & Boohaker, E. A. (1983) Cancer Res. 43, 2997-3000). As noted above, the elaboration of IL-4 by a tumor transplant induces a potent anti-tumor effect which is mediated by infiltrating eosinophils (Tepper, R. I., Pattengale, P. K. & Leder, P. (1989) Cell 57, 503-512; Tepper, R. I., Coffman, R. L. & Leder, P. (1992) Science 257, 548-551). We have also shown that eotaxin mRNA is induced at the site of the IL-4-transfected tumor cell transplant. Thus, eotaxin may be the critical eosinophil chemoattractant that, in part, mediates eosinophil tissue recruitment in this process. It is further conceivable that eotaxin is involved in other disease states mediated by IL-4 (e.g. asthma).

As we have noted, the mechanism of IL-4-mediated eosinophil accumulation in the tumor model and in allergic models is not completely understood. It is likely that IL-4 works in conjunction with other accessory cells to induce eosinophil recruitment. Consistent with this, IL-4 is known to induce the expression of the eosinophil endothelial cell adhesion receptor (VCAM-1) (Schleimer, R. P., Sterbinsky, S. A., Kaiser, J., Bickel, C. A., Klunk, D. A., Tomioka, K.,

Newman, W., Luscinskas, F. W., Gimbrone, M. A., Jr., McIntyre, B. W. & et al. (1992) J. Immun. 148, 1086-1092) and to promote the production of Th2 cells which elaborate IL-5. However, treatment of mice with neutralizing  
5 antibodies against VCAM-1 or IL-5 does not completely block eosinophil infiltration into tumor cells (Tepper, R. I., Coffman, R. L. & Leder, P. (1992) Science 257, 548-551; Tepper, R. I. (1994) J. All. Clin. Immun. 94, 1225-1231). In contrast, IFN- $\gamma$  production is increased in this process  
10 and treatment of mice with antibodies that neutralize IFN- $\gamma$  does prevent tumor killing Platzer, C., Richter, G., Uberla, K., Hock, H., Diamantstein, T. & Blankenstein, T. (1992) Eur. J. Immun. 22, 1729-1733. The cellular source of eotaxin mRNA found following IL-4 tumor cell transplantation  
15 is not known, but we have seen that treatment of endothelial cells with IFN- $\gamma$  induces expression of eotaxin mRNA. This raises the possibility that IL-4 may be inducing eotaxin mRNA by first inducing IFN- $\gamma$  in the IL-4 tumor model. The identification of endothelial cells and not mast cells as a  
20 cellular source of eotaxin has important implications for the design of therapeutic agents that would inhibit the production of eotaxin. These findings underscore the potential involvement of eotaxin in multiple immune responses, involving IL-4 and/or IFN- $\gamma$ .

25       The following examples are provided to illustrate not limit the invention.

#### EXAMPLES

##### I. MATERIALS AND METHODS

30       **Eotaxin Gene Analysis.** A cDNA probe from the coding region of guinea pig eotaxin cDNA (bp 57-356), (Rothenberg, M. E., Luster, A. D., Lilly, C. M., Drazen, J. M. & Leder, P. (1995) J. Exp. Med. 181, 1211-1216), was <sup>32</sup>P-labeled with

Klenow enzyme and used to screen  $1 \times 10^6$  plaques from a mouse 129SV genomic library (Stratagene, La Jolla, CA) under conditions of low stringency (hybridization in 0.6M NaCl, 80mM TrisCl, 4mM EDTA, 0.1% sodium pyrophosphate, 0.1% SDS, 5 10X Denhardt's solution (0.002% polyvinylpyrrolidone, 0.002% BSA, 0.002% Ficoll 400), 100mcg/ml denatured herring sperm DNA at 50°C and blots washed with 1X SSC, 0.05% SDS at 50°C). Two plaques hybridized strongly and were purified. The mouse genomic DNA was liberated from the phage DNA by 10 Not I digestion and sub-cloned into pBlue-Script SK II (Stratagene). Restriction mapping indicated that the two genomic clones had overlapping regions. A 1.4 kb EcoRV genomic fragment that hybridized with guinea pig eotaxin was subcloned into pBlue-Script KS II, and subjected to 15 automated sequencing on both strands using Applied Biosystems Instrumentation (model 373a) and the dye-terminator protocol. Sequence analysis was performed using software developed by the University of Wisconsin genetics computer group Altschul, S. F., Gish, W., Miller, 20 W., Myers, E. W. & Lipman, D. J. (1990) J. Mol. Biol. 215, 403-410.

Eotaxin chromosomal localization was determined by the analysis of polymorphism in the CA repeat found in intron 2. PCR primers flanking this repeat were constructed 25 (50 sense oligonucleotide: CACCCTGAAAGCCATAGTGT and 30 antisense oligonucleotide: TGTGTACCTGGGAAATTAG) and genomic DNA was amplified by PCR. Using these primers, a size polymorphism was identified between C57BL/6J and Mus. spretus DNA and a panel of DNA from 94 interspecific 30 backcrosses between (C57BL/6EiJ x SPRET/Ei)F1 x SPRET/Ei (BSS) was genotyped by PCR, (Rowe, L. B., Nadeau, J. H., Turner, R., Frankel, W. N., Letts, V. A., Eppig, J. T., Ko, M. S. H., Thurston, S. J. & Birkenmeier, E. H. (1994) Mamm.

Gen. 5, 253-274). The resulting segregation pattern was compared to the approximately 1500 loci previously typed in this cross (Rowe, L. B., Nadeau, J. H., Turner, R., Frankel, W. N., Letts, V. A., Eppig, J. T., Ko, M. S. H., Thurston, S. J. & Birkenmeier, E. H. (1994) Mamm. Gen. 5, 253-274). Gene order was determined by minimizing double crossovers.

**Eotaxin cDNA Analysis.** The 1.4 kb Eco RV genomic fragment was used to screen a mouse (C57BL/6 X CBA) lung Uni-Zap cDNA expression library (Stratagene) under conditions of high stringency (hybridization in 50% formamide, 10% dextran sulfate, 5X SSC, 1X Denhardt's solution, 1% SDS, 100 mcg/ml denatured herring sperm DNA, and 20mM Tris at 42°C and blots were washed with 0.2X SSC, 0.5% SDS at 65°C). Four positive plaques were identified, purified, and phagemids were prepared according to the instructions of the library manufacturer. The four inserts were completely sequenced on both strands by automated sequencing. Alignment analysis was determined by the Clustal method using MegAlign software (DNASTAR Inc) (Higgins, D. G. & Sharp, P. M. (1988) Gene 73, 237-244).

**RNA analysis.** An SV-40 immortalized murine endothelial cell line (O'Connell, K. A. & Edidin, M. (1990) J. Immun. 144, 521-525) was cultured in DMEM medium supplemented with 10% iron fortified calf serum with or without 200 U/ml recombinant murine IFN- $\gamma$  (Genentech, Inc., San Francisco). Mouse bone-marrow derived mast cells were obtained by culturing mouse bone marrow in the presence of 50% WEHI-3 conditioned medium for 4 weeks (Razin, E, Cordon-Cardo, C. & Good R.A. (1981) Proc Natl Acad Sci U.S.A. 78, 2559-2561). Cell lines (WEHI-3, P815, and RAW 264.7) were purchased from American Type Tissue Culture Collection (Rockville, MD). In the tumor transplantation experiments, 2 X 10<sup>6</sup> J558L or a murine IL-4 transfected

J558L cell line (I3L6) were injected s.c. into 4- 6 week old Balb/c female mice (Jackson Laboratory) as previously described (Tepper, R. I., Pattengale, P. K. & Leder, P. (1989) Cell 57, 503-512). At various times after tumor  
5 transplantation, the local skin was treated with Nair (Carter Products, N.Y.) to remove hair, and the skin and subcutaneous tissue was excised. RNA was isolated by CsCl centrifugation in guanidine isothiocyanate (Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979)  
10 Biochem. 18, 5294-5299 or by using RNazol (Biotecx Lab, Inc.)). 10 mcg of total RNA was fractionated by gel electrophoresis using 1.5% agarose and 1.9% formaldehyde, and transferred to Gene Screen (NEN Dupont) membranes. The murine MCP-1 probe is described in (Rollins, B. J.,  
15 Morrison, E. D. & Stiles, C. D. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 3738-3742). High stringency hybridization and washing was performed as described above. The quantification of total RNA was determined by hybridization of a ribosomal 28S cDNA probe (Rich, B. E. & Steitz, J. A.  
20 (1987) Mol. Cell. Biol. 7, 4065-4074). Quantitation of the intensity of band hybridization was determined using a Phosphor-Imager (Molecular Dynamics).

**Construction and Transfection of Eotaxin Expression Vectors.** PCR primers were designed to amplify the coding  
25 region of murine eotaxin flanked by convenient restriction sites for subsequent sub-cloning. PCR was performed under standard conditions using eotaxin cDNA-pBlueScript as a template. The resulting PCR products were subsequently subcloned using a TA cloning kit (Invitrogen, San Diego, CA)  
30 and confirmatory sequencing was performed. Eotaxin cDNA was subcloned into the Hind III/EcoRI sites of pcDNA-I/Amp (Invitrogen). 4 mcg of the eotaxin-pcDNA-I construct was transfected into 100 mm plates containing ~30% confluent COS

cells using DEAE-Dextran (Lopata, M. A., Cleveland, D. W. & Sollner-Webb, B. (1984) Nucl. Acids Res. 12, 5707-5717). In a replicate sample of COS cells, transfection efficiency was >50% using a CMV promoter-placental alkaline phosphatase control plasmid (Fields-Berry, S. C., Halliday, A. L. & Cepko, C. L. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 693-697). RNA expression was confirmed by Northern analysis using the murine eotaxin cDNA as a probe. Eotaxin-pcDNA-I transfected or mock transfected COS cell supernatant was obtained after 72 hrs of culture and stored at 4°C. In another set of transfection experiments, eotaxin was similarly subcloned into the HindIII/EcoRI site of MoLTR-SV40 I/PA expression vector as previously described (Luster, A. D. & Leder, P. (1993) J. Exp. Med. 178, 1057-1065). 20 mcg of linearized eotaxin-MoLTR construct and 1 mcg of linearized neomycin resistance plasmid pSV7Neo were used to transfect J558L cells by electroporation. G418 resistant cells from single wells were analyzed for eotaxin mRNA expression by Northern analysis. Cells expressing eotaxin or control untransfected cells (that do not express eotaxin) were expanded in large cultures. In order to optimize the concentration of eotaxin in the supernatant, the cells were grown at high density (1 X 10<sup>6</sup> cells/ml) in RPMI without FCS, cultured for 72 hrs, and the conditioned medium was concentrated 5-fold with Centricon 3000 microconcentrators (Amicon, Beverly, MA) before being stored at 4°C.

**Chemotaxis Assays.** Murine eosinophils were isolated from IL-5 transgenic mice (Dent, L. A., Strath, M., Mellor, A. L. & Sanderson, C. J. (1990) J. Exp. Med. 172, 1425-1431). These mice develop splenomegaly with eosinophils accounting for ~30% of the splenocytes. Eosinophils were purified from the spleen using

immuno-magnetic separation to remove the contaminating splenocytes. Briefly, splenocytes were labeled with anti-Thy-1 (M5/49), anti-B220 (6B2), and anti-Lyt-2 (53-6.7). Hybridoma cell lines were obtained from American Type Culture Collection and hybridoma cell supernatants were used as a source of antibodies. The antibody labeled cells were treated with sheep anti-rat serum coated-magnetic beads (M450, Dynal, Great Neck, NY) and eosinophils were enriched by negative selection through a magnetic field. The resulting eosinophil preparations were 85-92% pure. Macrophages cells were isolated from the peritoneal cavity of mice that had been pre-treated (2 days prior) with intraperitoneal injection of 2.9% thioglycollate (Difco, Detroit, CA). Peritoneal neutrophils were isolated from mice pre-treated with sodium casein (Luo, Y., Laning, J., Devi, S., Mak, J., Schall, T. J. & Dorf, M. E. (1994) J. Immun. 153, 4616-4624). Macrophages and neutrophils were purified by Percoll gradients (Luo, Y., Laning, J., Devi, S., Mak, J., Schall, T. J. & Dorf, M. E. (1994) J. Immun. 153, 4616-4624) and accounted for >90% of the cells. Eosinophils or macrophages were suspended in HBSS with 0.05% BSA at  $2 \times 10^6$  cells/ml, respectively, and 50 ml of replicate cells were placed in the top well of a 48 well micro-chemotaxis chamber (Neuro Probe, Inc, Cabin John, MD). A polycarbonate filter with 5- $\mu$ m pores was used to separate the cells from buffer (30 ml) alone or buffer containing recombinant murine MIP-1a (R&D Systems, Minneapolis, MN), PAF (Calbiochem, La Jolla, CA), COS cell supernatant, or J558L supernatant. Cells were incubated at 37°C for 60 minutes (eosinophils and neutrophils) or 90 minutes (macrophages) and the cells that migrated across the filter and adhered to the bottom side of the filter were stained



with Diff-Quick (Baxter Scientific, McGaw Park, IL). The number of cells per 400X field were counted.

#### Induction of Airway Inflammation. Airway

inflammation was induced in male Hartley guinea pigs

- 5 (300-500 g body weight) by OVA sensitization as previously reported Lilly, C.M., L. Kobzik, A.E. Hall, and J.M. Drazen. 1994. Effects of chronic airway inflammation on the activity and enzymatic inactivation of neuropeptides in guinea pig lungs. J. Clin. Invest. 93:2667-2674. Briefly,
- 10 guinea pigs were pretreated with pyrilamine malate by i.p. injection prior to aerosolized OVA (1% w/v in 0.9% sterile sodium chloride) or saline alone. Animals were exposed in an aerosol chamber on three occasions at 7-d intervals and the lungs were harvested at various points after the final
- 15 exposure. Organs were frozen in liquid nitrogen and used for subsequent RNA isolation. A guinea pig lung epithelial cell line (JH4 clone 1) and a guinea pig colon adenocarcinoma cell line (GPC-16) were purchased from American Type Tissue Culture Collection (CCL 158).

- 20 **Eotaxin cloning.** Based on the published amino acid sequence of guinea pig eotaxin Jose, P.J., D.A. Griffiths-Johnson, P.D. Collins, D.T. Walsh, R. Moqbel, N.F. Totty, O. Truong, J.J. Hsuan, and T.J. Williams. 1994.

- Eotaxin: a potent eosinophil chemoattractant cytokine
- 25 detected in a guinea pig model of allergic airways inflammation (J. Exp. Med. 179:881-887). The following degenerate oligonucleotide primers containing EcoR1 and BamH1 restriction sites respectively were synthesized: CCGGAATTCCA(CT)CC(AGC T)GG(AGCT)AT(ACT) (128 fold
- 30 degeneracy) and CGCGGATCCGC(AG)CA(AGT)AT CAT(CT)TT(AG)TC (32-fold degeneracy). First strand cDNA was synthesized from guinea pig lung RNA and PCR was performed with an initial five cycles at 37° for 60s, followed by 25 cycles at 50° for

50% formamide, 10% dextran sulfate, 5X SSC, 1X Denhardt's solution (0.0002% (w/v) polyvinylpyrrolidone, 0.0002% (w/v) BSA, 0.0002% (w/v) Ficoll 400), 1% (w/v) SDS, 100 mcg/ml denatured herring sperm DNA, and 20mM Tris at 42°C and blots were washed with 0.2X SSC, 0.5% SDS at 65°C. Low stringency hybridization was performed in 0.6M NaCl, 80mM TrisCl, 4mM EDTA, 0.1% (w/v) sodium pyrophosphate, 0.1% (w/v) SDS, 10X Denhardt's, 100mcg/ml denatured herring sperm DNA at 50°C and washed with 1XSSC, 0.05% SDS at 50°C. Quantitation of the intensity of band hybridization was determined using a Phosphor-Imager (Molecular Dynamics).

**Statistical Analysis.** The statistical significance of differences between means was determined by analysis of variance (ANOVA).  $P < 0.05$  was considered significant. When ANOVA indicated a significant difference, the Newman-Keuls test was used to determine which groups were significantly different from each other.

60s (denaturation at 95° for 30s and extension at 72° for 90s) in order to amplify a 130 bp eotaxin cDNA fragment that was subsequently subcloned into Bluescript II KS

(Stratagene). Construction of a cDNA library using poly A  
5 RNA isolated from the lung of an OVA sensitized guinea pig was performed using Stratagene ZAP Express Vector according to the directions of the manufacturer. 500,000 independent clones were subsequently amplified and an aliquot of this cDNA library containing 1 X 10<sup>6</sup> phage was screened with the  
10 130 bp eotaxin cDNA that had been 32P- labeled with Klenow enzyme. Two phagemids were isolated and subsequently subjected to automated sequencing on both strands using Applied Biosystems Instrumentation (model 373a) and the dye-terminator protocol. Sequence analysis was performed using  
15 software developed by the University of Wisconsin genetics computer group (Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman. 1990). Basic local alignment search tool (J. Mol. Biol. 215:403-410).

**DNA and RNA analysis.** RNA was isolated by CsCl  
20 centrifugation in guanidine isothiocyanate (Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter 1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease (Biochemistry. 18:5294-9). DNA was isolated from these gradients as well. In some  
25 cases, RNA was isolated using RNazol (Biotecx Lab, Inc.) according to the directions of the manufacturer. Poly A RNA was enriched by elution through an oligo dT column (Pharmacia). 10 mcg of total RNA, 2 mcg of poly A RNA, or 10 mcg of restriction endonuclease cut DNA was electrophoresed  
30 in agarose, and transferred to Gene Screen (NEN Dupont) membranes. Membranes were hybridized with 32P labeled full length cDNA or a fragment encoding the translated protein (bp 57-356). High stringency hybridization was performed in

## II. EOTAXIN GENOMIC STRUCTURE AND CHROMOSOMAL LOCALIZATION.

One way to clarify the question of whether guinea pig eotaxin is a homologue of a previously known chemokine is to shift the focus of attention to the mouse, in which a number of C-C chemokine genes are available for direct structural comparison. Accordingly, we used the coding region of guinea pig eotaxin cDNA and conditions of low stringency to screen a murine genomic library. Two overlapping clones were recovered and, from these, a 1.4 kb EcoRV genomic fragment was identified which hybridized to guinea pig eotaxin cDNA. Sequence analysis revealed that it encoded two exons with striking homology to guinea pig eotaxin (Fig. 1 and see below). Exon 1 mapped to the 5 kb EcoRI-XhoI fragment shown in Figure 1 and a predicted signal peptide cleavage site was identified in the 50 region of exon 2 (Fig. 1) (Von Heijne, G. (1983) Eur. J. Biochem. 133, 17-21).

The sequence of the cloned genomic fragment revealed a stretch of 18 CA repeats in the intron between exons 2 and 3 that facilitated the chromosomal mapping of this gene. PCR primers flanking the repeat were used to amplify genomic DNA from several strains of mice and revealed a PCR product of similar size in the 129SV, DBA/2J, and C57BL/6J genomes. In contrast, DNA from *Mus. spretus* amplified a larger PCR product indicating a dinucleotide polymorphism (Fig 2a). A panel of backcross DNA (F1 X SPRET/Ei) was subsequently used to map the eotaxin gene based on this CA repeat polymorphism (Rowe, L. B., Nadeau, J. H., Turner, R., Frankel, W. N., Letts, V. A., Eppig, J. T., Ko, M. S. H., Thurston, S. J. & Birkenmeier, E. H. (1994) Mamm. Gen. 5, 253-274). The eotaxin gene mapped to chromosome 11 between D11Mit markers 7 and 36 (Fig 2b). A comparison to the consensus map from

the Chromosome Committee (Figure 2b) revealed this to be the C-C chemokine gene locus containing other chemokines (designated small inducible cytokines, Scy, a1-6) Lossie, A. C., Macphee, M., Buchberg, A. M. & Camper, S. A. (1994) Mamm. Gen. 5, S164-180.

### III. ANALYSIS OF EOTAXIN CDNA

In order to obtain the complete coding sequence to the candidate eotaxin gene, the 1.4 kb EcoRV genomic fragment containing exons 2 and 3 was used in a high stringency screen of a mouse lung cDNA library. Four positive plaques were identified, purified, and sequenced (Figure 3a). The cDNA from the longest cDNA was 994 bp long with an open reading frame that encoded 97 amino acids. A consensus sequence for translation initiation was identified around the first ATG codon in the longest open reading frame Kozak, M. (1987) Nucl. Acids Res. 15, 8125-8148. The 5' region of the cDNA encoded a putative hydrophobic leader sequence whose cleavage site was predicted to occur between Ala and His (shown with the arrow in Figure 3). These residues are conserved in guinea pig eotaxin. The 30 untranslated region contained three OAUUUAO domains (shown with hatched lines). Sequences of this type have been reported to decrease the mRNA stability of other cytokine mRNAs (Shaw, G. & Kamen, R. (1986) Cell 46, 659-667) and are also present in guinea pig eotaxin (Rothenberg, M. E., Luster, A. D., Lilly, C. M., Drazen, J. M. & Leder, P. (1995) J. Exp. Med. 181, 1211-1216).

Examination of the nucleotide and protein sequences revealed that murine eotaxin was different than other sequenced murine chemokines and most homologous to guinea pig eotaxin (78% and 69% identical in bp and amino acids, respectively) (Table 1). Guinea pig and murine eotaxin are

most homologous to members of the MCP family.  
None-the-less, several features distinguish eotaxin from the  
MCP proteins (Figure 3b). The predicted mature eotaxin  
proteins both start with His-Pro, whereas MCP family members  
5 start with Gln-Pro. Further, the third amino acid in mature  
guinea pig and murine eotaxin is Gly which distinguishes it  
from the MCPs. Guinea pig and murine eotaxin also have a  
gap at amino acids positions six and seven in their mature  
proteins which distinguish them from the other C-C  
10 chemokines (shown with \*). As with all C-C chemokines,  
tyrosine28 is conserved in eotaxin Lusti-Narasimhan, M.,  
Power, C. A., Allet, B., Alouani, S., Bacon, K. B., Mermoud,  
J. J., Proudfoot, A. E. I. & Wells, T. N. C. (1995) J. Biol.  
Chem. 270, 2716-2721. Moreover, owing to the carboxy  
15 terminal end of eotaxin which is rich in basic amino acids  
(including the conservation of three consecutive Lys (shown  
with + symbol in Figure 3b), the predicted pI and charge at  
pH 7 is 10 and 12, respectively.

Table 1				
Comparison of Mouse Eotaxin Coding cDNA with other Chemokines				
	Chemokine	AA Similarity	AA Identity	BP Identity
5	gp Eotaxin*	69%	63%	78%
	mu MCP-1	64	49	63
	hu MCP-1	64	53	68
	gp MCP-1	60	45	60
	hu MCP-2*	60	49	ND
10	hu MCP-3*	67	57	71
	mu MCP3(MARC)	61	45	62
	hu MIP-1 $\alpha$ *	53	35	51
	hu MIP-1 $\beta$	51	34	52
	hu RANTES*	49	32	46
15	hu E309	44	32	49
	mu TCA3	44	25	45
	hu IL-8*	47	26	43

\*Chemokines indicated have been shown to be active towards human eosinophils. ND, not determined.

#### 20 IV. EOSINOPHIL CHEMOTAXIS BY EOTAXIN

Given the eosinophil chemoattractant activity of guinea pig eotaxin (Griffiths-Johnson, D. A., Collins, P. D., Rossi, A. G., Jose, P. J. & Williams, T. J. (1993) Biochem. Biophys. Res. Comm. 197, 1167-1172; Jose, P. J., Griffiths-Johnson, D. A., Collins, P. D., Walsh, D. T., Mogbel, R., Totty, N. F., Truong, O., Hsuan, J. J. & Williams, T. J. (1994) J. Exp. Med. 179, 881-887), it was essential to test this property using the putative mouse homologue. Accordingly, the chemoattractant activity of the murine eotaxin protein was examined by expressing murine eotaxin cDNA in two different eukaryotic expression systems and testing their products on mouse eosinophils in-vitro. First, stable transfectants of plasmacytoma cells (J558L) with a MuLV MoLTR- eotaxin construct were derived and grown in serum free media. Second, the eotaxin cDNA was

sub-cloned into the pcDNA-I/Amp plasmid and used for transient transfection of COS cells. The supernatants of these cells were used as a source of eotaxin protein.

Normal mice do not have appreciable numbers of eosinophils so murine eosinophils were purified from IL-5 transgenic mice. IL-5 is an eosinophil growth and activating factor and IL-5 transgenic mice have profound eosinophilia that facilitates their isolation (Dent, L. A., Strath, M., Mellor, A. L. & Sanderson, C. J. (1990) J. Exp. Med. 172, 1425-1431). Using a 48 well micro-chemotaxis chamber, murine eosinophils exhibited a strong chemotactic response to  $10^{-6}$ - $10^{-7}$  M PAF and 100-1000 ng/ml of recombinant murine MIP-1a, both positive controls (Fig 4a). The eotaxin J558L supernatant also demonstrated a strong eosinophil chemotactic activity compared to the negative control supernatant (Fig 4a). The possible inhibitory effect of the control supernatant may relate to the release of toxic metabolites during the harvesting of the cell supernatant. The supernatant from transfected COS cells also produced an increase in chemotaxis compared to mock-transfected COS cell supernatant (Fig 4b). The larger chemotactic response in Fig 4b compared with Fig 4a is likely due to the presence of FCS in the COS cell supernatants. In all experiments, migrating cells were >95% eosinophils. Eotaxin-COS cell and J558L cell supernatants had no activity on mouse macrophages or neutrophils whereas the macrophages had a strong chemotactic response to murine MIP-1a and the neutrophils had a strong chemotactic response to KC (data not shown).

#### 30 V. ANALYSIS OF EOTAXIN mRNA EXPRESSION IN MICE

Because clues to function can be inferred from the expression pattern of a gene, eotaxin mRNA was assessed in



various organs of normal mice. Analysis of an equal amount of total RNA by optical density and ethidium bromide staining, revealed easily detectable levels of eotaxin mRNA expression in skin, thymus, lymph node, mammary gland, skeletal muscle, and lower levels in the heart and lung (Figure 5). Other tissue with expression included the stomach, tongue, and one spleen sample (data not shown). This relative expression pattern varied somewhat between RNA preparations, probably reflecting strain and age variability in the mice. As predicted from the size of the cDNA, the mRNA transcript was approximately 1kb.

#### XI. REGULATION OF EOTAXIN mRNA EXPRESSION IN-VIVO BY IL-4

When IL-4 transfected tumor cells are transplanted into the skin of syngeneic or immunodeficient mice, there is a remarkable eosinophil infiltration within 18 hours (Tepper, R. I., Pattengale, P. K. & Leder, P. (1989) Cell 57, 503-512). These eosinophils have been shown to be critical for the anti-tumor effect of IL-4, though the mechanism of their recruitment is not understood. Eotaxin would be a logical candidate for this role (Tepper, R. I., Coffman, R. L. & Leder, P. (1992) Science 257, 548-551). To assess this possibility, eotaxin mRNA expression at the site of tumor cell transplantation was assayed at various times after transplantation of the IL-4-producing tumor cells. Within six hours, there was a marked increase in eotaxin mRNA compared with controls which consisted of transplanting untransfected tumor cells or untreated skin (Fig. 6a). This increase persisted for 7 days. Figure 6b shows representative data at the 24 hour time point for eotaxin mRNA in the skin of untransplanted mice and mice transplanted with IL-4-producing and non-producing tumor cells.

## VII. PRODUCTION OF EOTAXIN BY ENDOTHELIAL CELLS

Since endothelial cells are known to produce several chemokines including MCP-1 (Rollins, B. J. & Pober, J. S. (1991) Am. J. Path. 138, 1315-1319) that are increased by interferon- $\gamma$  (IFN- $\gamma$ ), the ability of IFN- $\gamma$  to induce eotaxin mRNA expression in endothelial cells was also examined. An SV40 immortalized murine endothelial cell line was treated with cytokine and total RNA was analyzed by RNA blot hybridization.

Eotaxin mRNA was undetectable in non-treated cells, but was detectable by 6 hrs of treatment with IFN- $\gamma$  (Fig. 6c) and returned to an undetectable level by 18 hrs. As a control, murine macrophage chemoattractant protein-1 (MCP-1) mRNA was found to be readily detectable constitutively and to be increased by IFN- $\gamma$  treatment (Fig. 6c) as previously reported (36). Eotaxin mRNA expression in mast cells and macrophages was also examined. Whereas the macrophage cell line, RAW 267.4, had easily detectable levels of murine MCP-1 mRNA which increased after 18 hrs of IFN- $\gamma$ , both untreated and treated cells had no eotaxin mRNA detectable (data not shown). Similarly, a mouse mast cell line (P815) and Con-A activated mouse bone marrow derived mast cells had easily detectable expression of MCP-1 mRNA, but no detectable expression of eotaxin mRNA (data not shown).

## VIII. ANALYSIS OF GUINEA PIG EOTAXIN cDNA.

Using degenerate oligonucleotide primers based upon the amino acid sequence of guinea pig eotaxin, a 130 bp cDNA was amplified by PCR from guinea pig lung single stranded cDNA. This PCR product encoded a peptide identical to eotaxin and was used to screen an amplified cDNA library made from the inflamed lung of an OVA-sensitized guinea pig. From sixty positive plaques, six plaques were subsequently purified and their excised phagemids had an insert size of

~700-800 bp. Sequence analysis of the longest two inserts revealed that the regions of overlap were identical. The cDNA was 818 bp long with an open reading frame that encoded 96 amino acids. The predicted protein sequence of the carboxy terminal 73 amino acids agreed exactly with the protein sequence of eotaxin isolated from guinea pig bronchoalveolar fluid (shown underlined in Figure 7) except for three amino acids that were previously ambiguous (shown surrounded by a box in Figure 7) (Jose, P.J., D.A. Griffiths-Johnson, P.D. Collins, D.T. Walsh, R. Moqbel, N.F. Totty, O. Truong, J.J. Hsuan, and T.J. Williams 1994 J. Exp. Med. 179:881-887).

The 5' region of the cDNA encoded a putative hydrophobic leader sequence whose cleavage site was predicted to occur at the amino-terminal site at which the active eotaxin protein sequence starts (shown with the arrow in Figure 7) (Von Heijne, G. 1983 Eur. J. Biochem. 133:17-21). This structure strongly suggested that eotaxin was unlikely to exist as a precursor protein requiring additional proteolytic cleavage for activation. This type of biochemical processing has been seen with the platelet basic proteins, members of the C-X-C chemokine family (Holt, J.C, M.E. Harris, A.M. Holt, E. Lange, A. Henschen, and S. Niewiarowski. 1986 Biochemistry. 25:1988-96). A Kozak consensus sequence for translation initiation was identified 5' of the AUG (Kozak, M. 1987 Nucleic Acids Res. 15:8125-48). The 30 untranslated region encoded a mRNA with 59% AU nucleotides including two OAUUUAO domains (shown with the hatched lines) that have been reported to decrease the mRNA stability of other cytokine mRNAs (Shaw, G. and R. Kamen. 1986 Cell 46:659-67).

The nucleotide sequence showed significant homology to other members of the C-C chemokine family, in particular

to members of the MCP family (Van, D.J., P. Proost, J.P. Lenaerts, and G. Opdenakker 1992 J. Exp. Med. 176:59-65). The full length cDNA was 61, 58, 42, 38, and 52% identical in nucleic acid to human MCP-3, MCP-1, MIP-1a, RANTES, and guinea pig MCP-1, respectively. Even greater homology was seen when only the region of the cDNA that encoded protein was compared (Table 1). Comparison of the leader sequence with other sequences in the gene data bank revealed that the eotaxin leader sequence was homologous only to other leader sequences of the MCP family (81 and 74% nucleotide identity and 78 and 70% amino acid identity to human MCP-3 and MCP-1, respectively). This level of homology suggested that these leader sequences may have an additional biological role, perhaps in cellular targeting (e.g. localization to a granule sub-compartment). Comparison of the homology to other C-C chemokines revealed that the nucleotide identity was almost always greater than the amino acid identity and similarity (Table 2). Although the MCPs were initially characterized by their ability to activate and attract macrophages, MCP-3 has been reported to cause eosinophil chemotaxis (Dahinden, C.A., T. Geiser, T. Brunner, V. Vontscharner, D. Caput, P. Ferrara, A. Minty, and M. Baggiolini 1994 J. Exp. Med. 179:751-756; Biochem. Biophys. Res. Commun. 201:493-499).

#### IX. EOTAXIN GENE IN THE GUINEA PIG AND MOUSE GENOME.

Restriction endonuclease treatment of guinea pig DNA (Figure 8, lanes a&b) with analysis by Southern blotting under conditions of low stringency, revealed a single hybridizing band. Mouse genomic DNA cut with EcoRV (Figure 8, lane c) also revealed a single hybridizing band. This data suggests that a single gene encodes guinea pig eotaxin and suggests the existence of a closely related gene in the mouse.

#### Eotaxin mRNA expression in different organs.

Northern blot analyses of total RNA isolated from different guinea pig tissue samples revealed easily detectable constitutive expression of eotaxin in the lung (Figure 9).

- 5 The predominant hybridizing band had a size of ~0.8 kb. Other hybridizing bands were not detectable using poly A selected RNA. Lower levels were detectable in the intestines, stomach, heart, thymus, spleen, liver, testes, and kidney. In these latter tissues, eotaxin mRNA was more  
10 easily detected on poly A blots. The intestine showed some variability in expression between two different animals (Figure 9). In addition, no RNA was detectable in the brain, bone marrow, or skin. Likewise, macrophages isolated and cultured from the spleen, a lung epithelial cell line, and a  
15 colon adenocarcinoma cell line were not found to express eotaxin mRNA. The finding of constitutive eotaxin mRNA in mucosal tissues wherein eosinophils predominantly reside (lung and intestines) suggests that eotaxin may play a role in the normal tissue homing and turnover of eosinophils.

#### 20 X. THE INDUCTION OF EOTAXIN mRNA IN ALLERGIC AIRWAY INFLAMMATION.

- Since eotaxin mRNA was found to be expressed at relatively high levels in the lung of healthy guinea pigs, it was important to determine if this mRNA could account for  
25 all the protein released following allergen challenge, or whether eotaxin mRNA levels also increased. Therefore guinea pigs were sensitized to either aerosolized OVA or exposed to saline twice at a 7 day interval. Following a third dose of aerosolized antigen, the lungs of sensitized, but not saline  
30 exposed, guinea pigs develop a mucosal and submucosal eosinophil infiltration. The inflammation is most prominent at 17 hrs and persists for at least 3 days (Hutson, P.A., M.K. Church, T.P. Clay, P. Miller, and S.T. Holgate. 1988.

Am. Rev. Respir. Dis. 137:548-57; Lilly, C.M., L. Kobzik, A.E. Hall, and J.M. Drazen 1994 J. Clin. Invest. 93:2667-2674). At various times after antigen challenge, poly A selected RNA were isolated from replicate lungs and equal amounts were examined by Northern blot analysis for eotaxin mRNA expression. Eotaxin mRNA levels increased ~6-fold ( $p < 0.01$ ) by 3 hrs compared with the lungs of saline treated guinea pigs (Fig. 10). All other time points were not significantly different (Fig. 11).

The cloning of guinea pig eotaxin cDNA has allowed us to make several observations relevant to the biology of eotaxin. The eotaxin gene is expressed at relatively high levels in the lungs of healthy guinea pigs without airway inflammation. In contrast, the chemotactic activity ascribed to eotaxin has been reported to be undetectable in the bronchoalveolar fluid of non- antigen challenged guinea pigs (Jose, P.J., D.A. Griffiths-Johnson, P.D. Collins, D.T. Walsh, R. Moqbel, N.F. Totty, O. Truong, J.J. Hsuan, and T.J. Williams 1994 J. Exp. Med. 179:881-887). Thus, eotaxin mRNA is expressed at easily detectable constitutive levels in the lung when eotaxin activity is undetectable. This presents several alternative possibilities regarding the eotaxin protein: (1) it is rapidly degraded; (2) it is expressed at a low level which previously was not detectable; (3) it remains in an inaccessible location (e.g. a mast cell granule) and/or is biologically inhibited until after antigen challenge; (4) it requires additional biochemical processing for activation or some combination of the above. It is unlikely that eotaxin requires additional processing for activation since the cDNA structure predicts that active eotaxin is generated directly after removal of the leader sequence. It is interesting that the lungs of healthy guinea pigs without eosinophilic inflammation have

detectable eosinophils in the bronchoalveolar fluid at baseline (Hutson, P.A., M.K. Church, T.P. Clay, P. Miller, and S.T. Holgate 1988 Am. Rev. Respir. Dis. 137:548-57). Low levels of eotaxin protein may regulate basal eosinophil tissue homing.

Following antigen challenge, eotaxin gene expression in the lung is further increased during the early part of the late phase response. This change in mRNA parallels the peak changes in eotaxin protein release into the bronchoalveolar fluid which also peaks at 3 hours (Jose, P.J., D.A. Griffiths-Johnson, P.D. Collins, D.T. Walsh, R. Moqbel, N.F. Totty, O. Truong, J.J. Hsuan, and T.J. Williams 1994 J. Exp. Med. 179:881-887). Only antigen exposure is associated with eosinophilic airway inflammation and bronchial hyper-responsiveness (Hutson, P.A., M.K. Church, T.P. Clay, P. Miller, and S.T. Holgate 1988 137:548-57; Sanjar, S., S. Aoki, A. Kristersson, D. Smith, and J. Morley 1990 J. Pharmacol. 99:679-86). Thus, up-regulation of gene expression, and not constitutive gene expression, is associated with the pathogenesis of airway disease. Eotaxin is likely to work in parallel with other cytokines generated during the late phase response. For example, IL-5, a cytokine produced during the late phase response, can prime eosinophils to respond to another C-C chemokine, RANTES, and can promote eosinophil tissue survival and activation (Rothenberg, M.E., J. Petersen, R.L. Stevens, D.S. Silberstein, D.T. McKenzie, K.F. Austen, and W.J. Owen 1989 J. Immunol. 143:2311-6; Ebisawa M., T. Yamada, C. Bickel, D. Klunk, and R.P. Schleimer 1994 J. Immunol. 153: 2153-60).

Examination of RNA samples from multiple tissues for the expression of eotaxin mRNA reveals that in addition to the lung, lower levels are seen in a variety of other tissues. This suggests a more widespread function for this

molecule. With the development of immunological reagents to detect the eotaxin protein, it will be important to compare protein and mRNA expression in these tissues. Finally, this cDNA enables the identification of analogous genes in other species and the development of molecular and immunological tools to examine the role of this molecule in allergic models and human disease. For example, Fig. 14 provides the human eotaxin sequence.

#### SEQUENCE DEPOSITS

The murine sequence data reported herein has been deposited in the GenBank data base (accession no. U26426) and the chromosomal mapping data has been deposited in the Mouse Genome Data Base (accession no. MGD-CREX-329).

#### OTHER EMBODIMENTS

In other embodiments, the invention includes any protein which is substantially identical to an eotaxin polypeptide (Figs. 3A and 12, preferably the sequence of Fig. 12); such homologs include other substantially pure naturally occurring mammalian eotaxin proteins as well as allelic variations; natural mutants; induced mutants; proteins encoded by DNA that hybridizes to the eotaxin sequences of Figs. 3A and 12 (preferably, the sequence of Fig. 12) under high stringency conditions or low stringency conditions (e.g., washing at 2X SSC at 40°C with a probe length of at least 40 nucleotides); and polypeptides or proteins specifically bound by antisera directed to a eotaxin polypeptide, especially by antisera to the active site or to the Max binding domain of an eotaxin protein. The term also includes chimeric polypeptides that include an eotaxin fragment.



The invention further includes analogs of the eotaxin polypeptide. Analogs can differ from the naturally occurring eotaxin protein by amino acid sequence differences, by post-translational modifications, or by both. Analogs of the invention will generally exhibit at least 70%, more preferably 80%, even more preferably 90%, and most preferably 95% or even 99%, identity with all or part of a naturally occurring eotaxin sequence. The length of comparison sequences will be at least 8 amino acid residues, preferably at least 24 amino acid residues, and more preferably more than 35 amino acid residues. Modifications include *in vivo* and *in vitro* chemical derivatization of polypeptides, e.g., acetylation, carboxylation, phosphorylation, or glycosylation; such modifications may occur during polypeptide synthesis or processing or following treatment with isolated modifying enzymes. Analogs can also differ from the naturally occurring eotaxin polypeptide by alterations in primary sequence. These include genetic variants, both natural and induced (for example, resulting from random mutagenesis by irradiation or exposure to ethanemethylsulfate or by site-specific mutagenesis as described in Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989, hereby incorporated by reference; or Ausubel et al., supra, hereby incorporated by reference). Also included are cyclized peptides molecules and analogs which contain residues other than L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g.,  $\beta$  or  $\gamma$  amino acids.

In addition to full-length polypeptides, the invention also includes eotaxin polypeptide fragments. As used herein, the term "fragment" means at least 10 contiguous amino acids, preferably at least 30 contiguous

amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least 60 to 80 or more contiguous amino acids. Fragments of eotaxin can be generated by methods known to those skilled in the art or may result from normal protein processing (e.g., removal of amino acids from the nascent polypeptide that are not required for biological activity or removal of amino acids by alternative mRNA splicing or alternative protein processing events).

Preferable fragments or analogs according to the invention are those which exhibit biological activity (for example, the ability to act as an eosinophil chemoattractant as described herein). Preferably, an eotaxin polypeptide, fragment, or analog exhibits at least 10%, more preferably 30%, and most preferably, 70% or more of the biological activity of a full length naturally occurring human murine eotaxin polypeptide.

MURINE EOTAXIN: AN EOSINOPHIL CHEMOATTRACTANT  
INDUCIBLE IN ENDOTHELIAL CELLS AND IN  
IL-4-INDUCED TUMOR SUPPRESSION

Abstract of the Disclosure

5        Disclosed is substantially pure murine and human  
eotaxin DNA sequences and eotaxin polypeptide, and methods  
of using such DNA and polypeptide to direct chemotaxis of  
eosinophils. Methods are provided for the diseases and  
disorders such as inflammation and tumorigenesis.

10    131086.B11

Applicant or Patentee: Andrew D. Luster et al.  
Serial or Patent No.: 60/000,449  
Filed on: June 22, 1995  
For: EOTAXIN: AN EOSINOPHIL CHEMOATTRACTANT



VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: President and Fellows of Harvard College  
Address of Organization: 17 Quincy Street, Cambridge, MA 02138  
Type of Organization:

- ☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION  
☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))  
☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA  
(NAME OF STATE: )  
(CITATION OF STATUTE: )  
☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF  
LOCATED IN THE UNITED STATES OF AMERICA  
☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF  
AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA  
(NAME OF STATE: )  
(CITATION OF STATUTE: )

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled EOTAXIN: AN EOSINOPHIL CHEMOATTRACTANT by inventor(s) Andrew D. Luster, Philip Leder, Marc Rotherberg and Eduardo Garcia described in

- ☐ the specification filed herewith.  
☒ application serial no. 60/000,449, filed June 22, 1995.  
☐ patent no. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

\*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name: The General Hospital Corporation

Address: 55 Fruit Street, Boston, MA 02114

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☒ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name: Joyce Brinton  
Title: Director, Office of Technology & Trademark Licensing  
Address: 124 Mt. Auburn St., University Place, Cambridge, MA 02138  
Signature: [Signature] Date: 10/19/95

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ATTORNEY DOCKET NO. 00383/025001



Applicant or Patentee: Andrew D. Luster et al.  
Patent No.: 60/000,449  
Filed or Issued: June 22, 1995  
EOTAXIN: AN EOSINOPHIL CHEMOATTRACTANT

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
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LOCATED IN THE UNITED STATES OF AMERICA  
☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF  
AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA  
(NAME OF STATE: )  
(CITATION OF STATUTE: )

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled EOTAXIN: AN EOSINOPHIL CHEMOATTRACTANT by inventor(s) Andrew D. Luster, Philip Leder, Marc Rothenberg and Eduardo Garcia described in

- ☐ the specification filed herewith.  
☒ application serial no. 60/000,449, filed June 22, 1995.  
☐ patent no. , issued .

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Full Name: President and Fellows of Harvard College

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

DAVID J. GLASS, Ph.D.  
ASSOCIATE DIRECTOR FOR PATENTS  
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Signature: [Signature] Date: Oct. 16, 1995



PATENT  
ATTORNEY JACKET NO: 00383/025001

### COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled EOTAXIN: AN EOSINOPHIL  
CHEMOATTRACTANT, the specification of which

☐ is attached hereto.

☒ was filed on June 22, 1995 as Application Serial No. 60/000,449  
and was amended on \_\_\_\_\_.

☐ was described and claimed in PCT International Application No. \_\_\_\_\_  
filed on \_\_\_\_\_ and as amended under PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Paul T. Clark, Reg. No. 30,162; Kristina Bieker-Brady, Reg. No. 39,109; William E. Booth, Reg. No. 28,933; Margaret A. Boulware, Reg. No. 28,708; Karl Bozicevic, Reg. No. 28,807; Barry E. Bretschneider, Reg. No. 28,055; Peter J. Devlin, Reg. No. 31,753; William J. Egan, Reg. No. 28,411; Willis M. Ertman, Reg. No. 18,658; David L. Feigenbaum, Reg. No. 30,378; Janis K. Fraser, Reg. No., 34,819; John W. Freeman, Reg. No. 29,066; Timothy A. French, Reg. No. 30,175; Alan H. Gordon, Reg. No. 26,168; Scott C. Harris, Reg. No. 32,030; Mark J. Hebert, Reg. No., 31,766; Gilbert H. Hennessey, Reg. No. 25,759; Charles Hicken, Reg. No. 18,411; Robert E. Hillman, Reg. No. 22,837; John F. Land, Reg. No. 29,554; G. Roger Lee, Reg. No. 28,963; Steven E. Lipman, Reg. No. 30,011; Gregory A. Madera, Reg. No. 28,878; Ralph A. Mittelberger, Reg. No. 33,195; Ronald E. Myrick, Reg. No. 26,315; Robert C. Nabinger, Reg. No., 33,431; Frank P. Porcelli, Reg. No. 27,374; Eric L. Prael, Reg. No. 32,590; Alan D. Rosenthal, Reg. No. 27,833; Richard M. Sharkansky, Reg. No. 25,800; John M. Skenyon, Reg. No. 27,468; Michael O. Sutton, Reg. No. 26,675; Reginald J. Suyat, Reg. No. 28,172; Rene D. Tegtmeyer, Reg. No. 33,567; Hans R. Troesch, Reg. No. 36,950; John R. Wetherell, Jr., Reg. No. 31,678; Wayne E. Willenberg, Reg. No. 28,488; John N. Williams, Reg. No. 18,948; Gary A. Walpert, Reg. No. 26,098; Dorothy P. Whelan, Reg. No., 33,814; and Charles C. Winchester, Reg. No. 21,040.

Address all telephone calls to Kristina Bieker-Brady at telephone number 617/542-5070.

Address all correspondence to Paul T. Clark, Fish & Richardson P.C., 225 Franklin Street, Boston, MA 02110-2804.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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148711.B11



COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled EOTAXIN: AN EOSINOPHIL  
CHEMOATTRACTANT, the specification of which

☐ is attached hereto.

☒ was filed on June 22, 1995 as Application Serial No. 60/000,449  
and was amended on \_\_\_\_\_

☐ was described and claimed in PCT International Application No. \_\_\_\_\_  
filed on \_\_\_\_\_ and as amended under PCT Article 19 on \_\_\_\_\_.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

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Figure 1

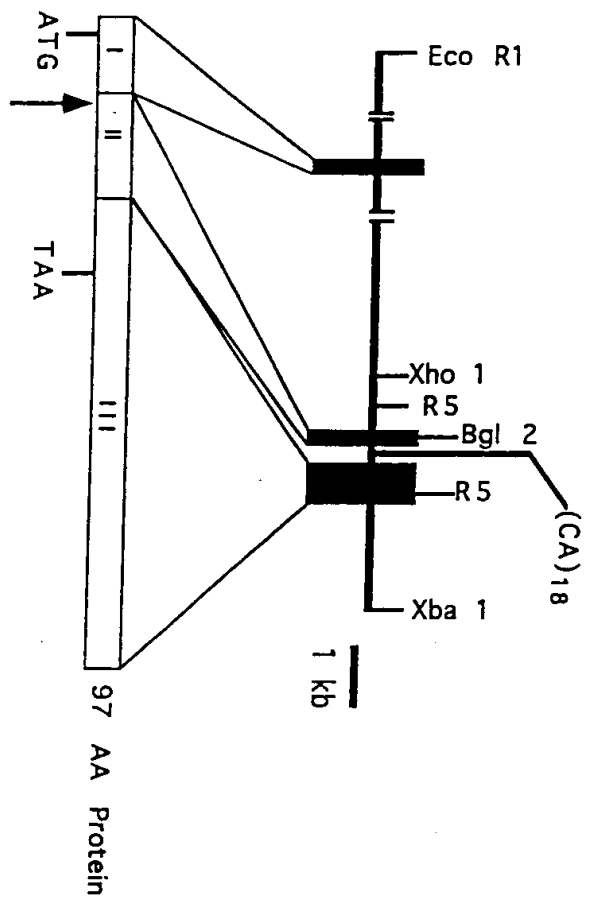
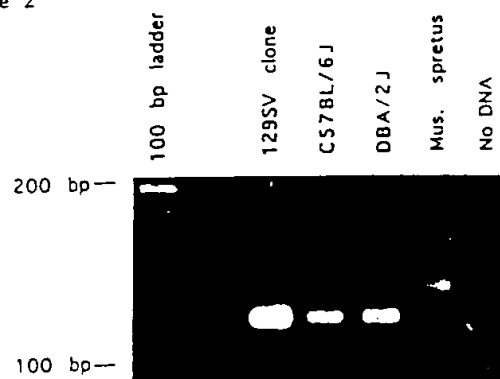


Figure 2

**A**



**B**

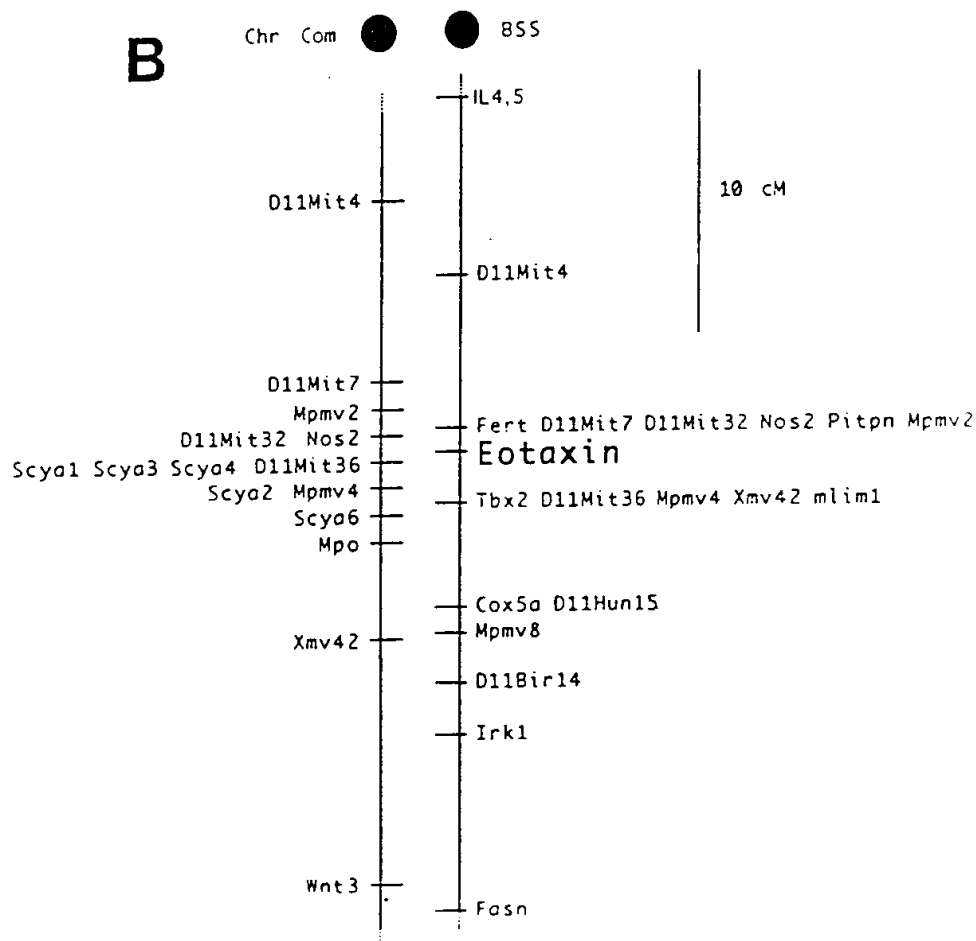
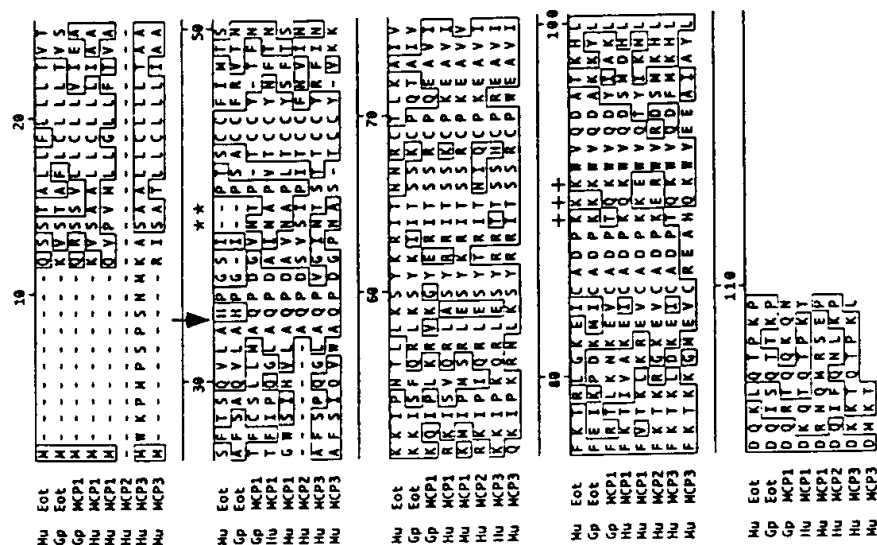


Figure 3

B



A

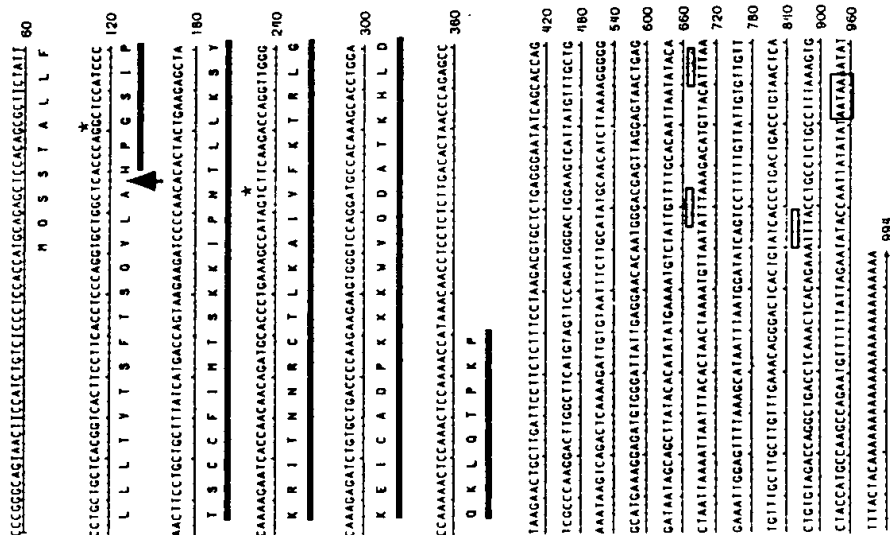


Figure 4

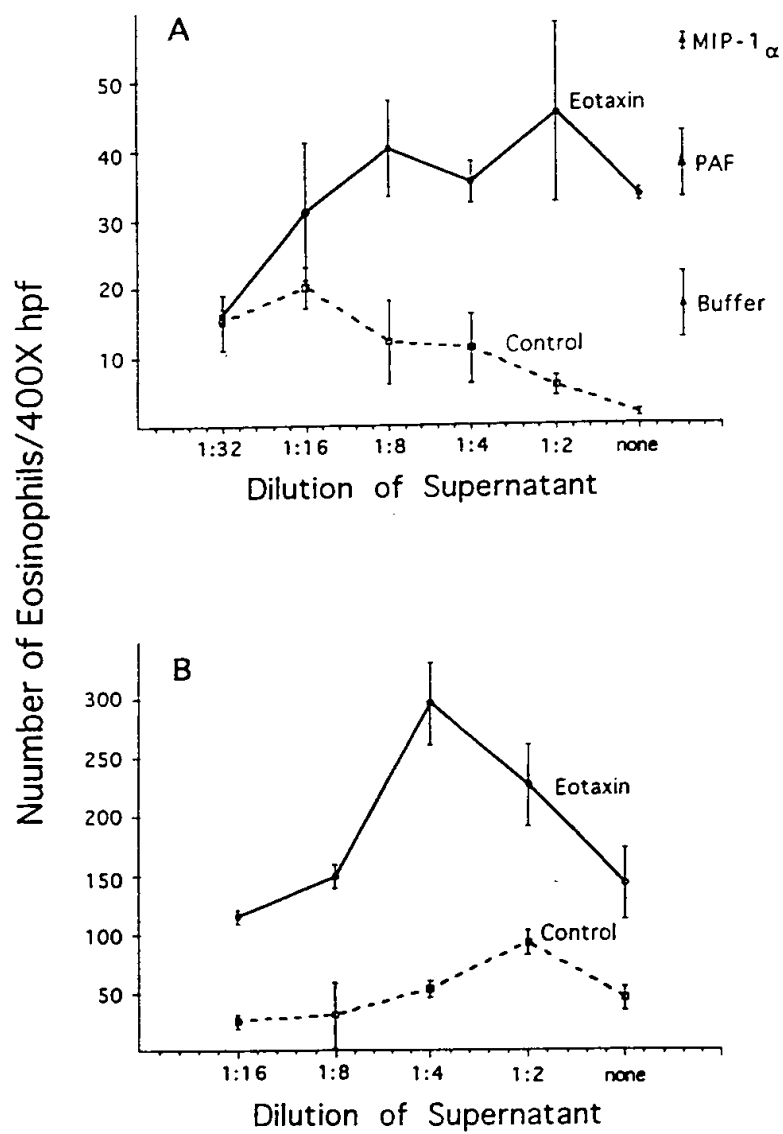


Figure 5

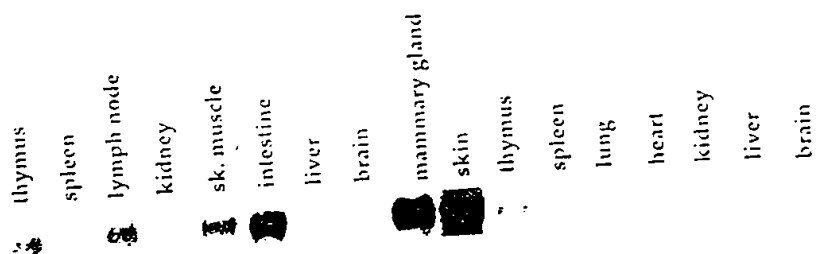


Figure 6

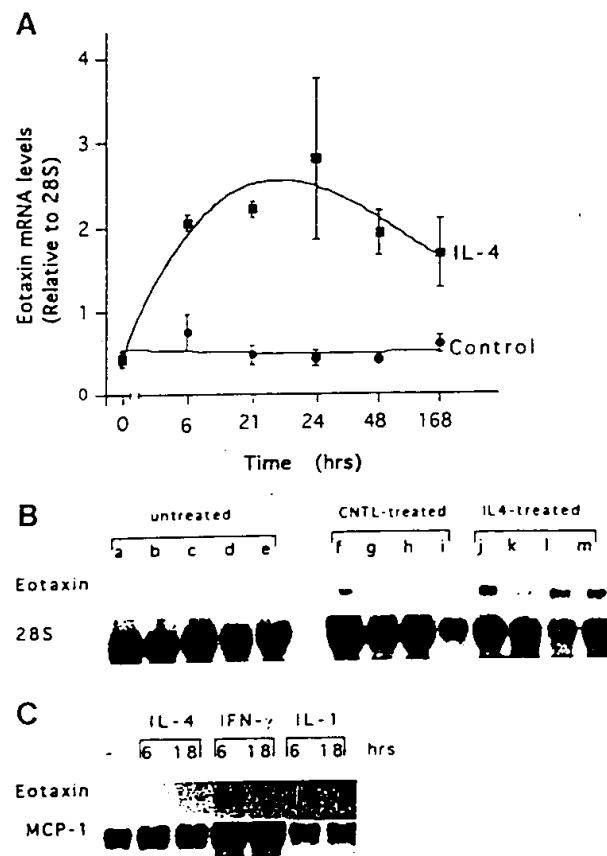


Figure 7

818



Figure 8

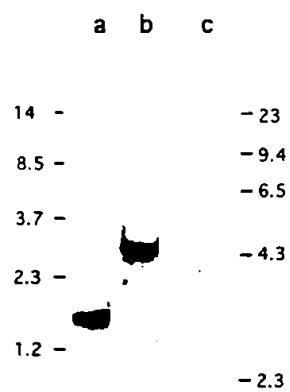


Figure 9

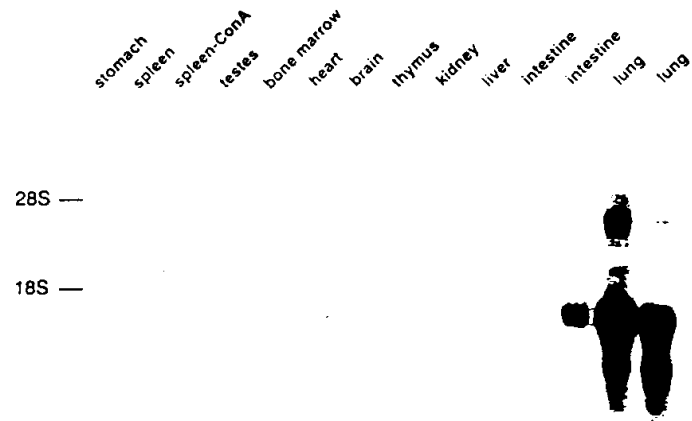


Figure 10

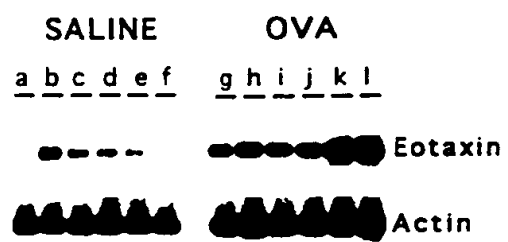


Figure 11

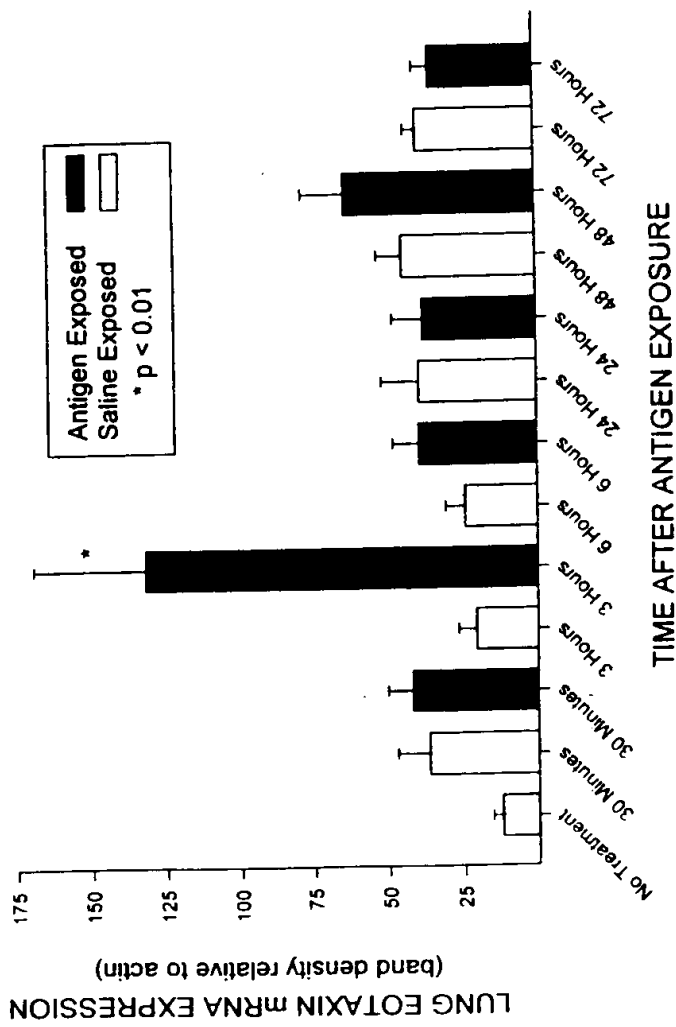


FIGURE 12

HUMAN EOTAXIN

Amino acid sequence:

H R X S V P T T C C F N L A N R K I P L Q R I E S Y R R I T S G K C P Q K A V I F K T K L A K D I C A D P K K K W V Q D S M K Y I D Q K S P T P K P

X = absent, A, S, T, or G.

FIGURE 13

HPG-IPSAACFRVTNKKISFQRLKSYKIITSSKCPQYAI VFEIKODKMICADPKKKWQDAKKYLDQISQTTK ~ G.Pig  
 HPGSIPTSCCFIWTSKKI PNTLLKSYKRIYNNRCTLKAIVFKIRLGKEICADPKKKWQDATKHLQKLOTPK ~ Munka  
 HPGSVPTTCCFNLANRKI PLQRLSYRRITSGKCPQKAVI FKTCLKADI CADPKKKWQDSRKTLDQKSPTPK ~ Human



# CLUSTAL W (1.82) Multiple Sequence Alignments

Sequence format is Pearson

**Sequence 1: 223187\_PanecI** 97 aa

**Sequence 2: LUSTER `449(FIG13)** 73 aa

Start of Pairwise alignments

Aligning...

Sequences (1:2) Aligned. Score: 94

Guide tree file created: [baaNRaOpr.dnd]

Start of Multiple Alignment

There are 1 groups

Aligning...

Group 1: Sequences: 2 Score:1544

Alignment Score 426

CLUSTAL-Alignment file created [baaNRaOpr.aln]

CLUSTAL W (1.82) multiple sequence alignment

```
223187_PanecI      MKVSAALLWLLLIAAAFSPQGLTGPASVPTTCCFNLNRKIPLQRLESYRRITSGKCPQK
LUSTER `449(FIG13) -----HPXSVPTTCCFNLNRKIPLQRLESYRRITSGKCPQK
                      * *****
```

```
223187_PanecI      AVIFKTKLAKDICADPKKKWVQDSMKYLDQKSPTPKP
LUSTER `449(FIG13) AVIFKTKLAKDICADPKKKWVQDSRKTLDQKSPTPK-
                      *****
```

Reference: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Query= Luster '449 (FIG13) (73 letters)

Database: genpept131

1,135,942 sequences; 348,344,575 total letters

Searching.....done

Sequences producing significant alignments:		Score (bits)	E Value
g2088509	eotaxin precursor [Homo sapiens]	146	6e-35
g17389652	small inducible cytokine subfamily A (Cys-Cys), mem	146	6e-35
g1552241	eotaxin [Homo sapiens]	146	6e-35
g1280141	eotaxin precursor [Homo sapiens]	146	6e-35
g1185440	eotaxin precursor [Homo sapiens]	146	6e-35
g2462478	eotaxin [Homo sapiens]	145	1e-34
g1531983	CC-chemokine [Homo sapiens]	145	1e-34
g22074367	eotaxin [Macaca mulatta]	134	3e-31
g6468531	eotaxin [Equus caballus]	110	3e-24
g2306775	eotaxin [Rattus norvegicus]	108	2e-23

>g2088509 eotaxin precursor [Homo sapiens]  
Length = 97

Score = 146 bits (368), Expect = 6e-35  
Identities = 69/72 (95%), Positives = 69/72 (95%)

Query: 2 PXSVPPTCCFNLNRKIPLQRLESYRRITSGKCPQKAVIFKTKLAKDICADPKKKWVQDS 61  
P SVPTTCCFNLNRKIPLQRLESYRRITSGKCPQKAVIFKTKLAKDICADPKKKWVQDS  
Sbjct: 25 PASVPTTCCFNLNRKIPLQRLESYRRITSGKCPQKAVIFKTKLAKDICADPKKKWVQDS 84

Query: 62 RKTLDQKSPTPK 73  
K LDQKSPTPK  
Sbjct: 85 MKYLDQKSPTPK 96

>g17389652 small inducible cytokine subfamily A (Cys-Cys), member  
11 (eotaxin) [Homo sapiens]  
Length = 97

Score = 146 bits (368), Expect = 6e-35  
Identities = 69/72 (95%), Positives = 69/72 (95%)

Query: 2 PXSVPPTCCFNLNRKIPLQRLESYRRITSGKCPQKAVIFKTKLAKDICADPKKKWVQDS 61  
P SVPTTCCFNLNRKIPLQRLESYRRITSGKCPQKAVIFKTKLAKDICADPKKKWVQDS  
Sbjct: 25 PASVPTTCCFNLNRKIPLQRLESYRRITSGKCPQKAVIFKTKLAKDICADPKKKWVQDS 84

Query: 62 RKTLDQKSPTPK 73  
K LDQKSPTPK  
Sbjct: 85 MKYLDQKSPTPK 96